JOURNAL OF BACTERIOLOGY, Apr. 1973, p. 239-248

Vol. 114, No. 1
Printed in U.S.A.

Organic Solvents as Probes for the Structure and Function of the Bacterial Membrane: Effects of Ethanol on the Wild Type and an Ethanol-Resistant Mutant of *Escherichia coli* K-12

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Received for publication 1 December 1972

The effects of ethanol on the growth of a wild-type *Escherichia coli* K-12 are described. These effects include a reduction of the steady-state growth rate and an interference with the division process. They appear as an immediate response to the addition of ethanol and are rapidly reversed by removal of ethanol. Mutants were selected that could grow at a concentration of ethanol that stopped wild-type growth. The growth of one of the mutants we studied (strain S9L100) is stimulated by the presence of ethanol, methanol, or dimethyl sulfoxide. This strain exhibits pleiotropic growth defects including abnormal cell division and morphology. It also appears to have an altered *lac* permease function which is not due to a mutation in the *Y* gene itself. We conclude that this mutant has an altered membrane and that the membrane defect may be the cause of the abnormal growth properties. The use of compounds which serve as general membrane perturbants and mutants resistant to these perturbants form a system accessible to both genetic and physical-biochemical techniques.

This report presents the initial results of a study intended to provide insight into the structural organization of the bacterial cytoplasmic membrane and the role of the membrane in organizing biological activity. Our approach is based upon the following view. The membrane consists of a lipid phase which solubilizes, to varying degrees, the biologically active components of the membranes (e.g., transport systems, cytochromes, etc.). The biological activities of these "solute" components are determined by the interaction of these components with the lipid and aqueous phases. These interactions can range from a specific requirement for some lipid component (e.g., as a cofactor) to the general requirement for a particular "solvent" environment to maintain the biologically active conformations or aggregations. In such a situation, perturbing the organization of the lipid phase should lead to altered structural relationships and possibly altered functional activity of the solute components.

We have used organic solvents to perturb bacterial functions, paying attention to those perturbations apparently resulting from effects on the membrane. Bacterial mutants resistant to specific effects have been isolated and thus we have a system accessible to both genetic and physical-biochemical techniques.

Our initial studies are concerned with ethanol. Ethanol is a member of a large class of amphiphilic compounds which serve as general anesthetics; an extensive literature exists on the evidence that anesthetics serve as general membrane perturbants (8, 11, 15). In this communication we describe the effect of ethanol on wild-type bacteria and some properties of a mutant whose growth is stimulated by ethanol. We present evidence that the ethanol-stimulated mutant has an altered membrane.

MATERIALS AND METHODS

Chemicals. Ortho-nitrophenyl-β-D-galactopyranoside (OMPG) was obtained from Pierce Chemical Co., Rockford, Ill. β-D-Galactosyl-1-thio-β-D-galactoside (TDG) and isopropyl-thio-β-D-galactoside (IPTG) were obtained from Cyclo Chemical, Los

Angeles, Calif. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. Dithiothreitol (Cleland's reagent) was from Calbiochem, Los Angeles, Calif. Formaldehyde (36.6% solution, analytical reagent grade) and methyl alcohol (anhydrous, spectrophotometric grade) were from Mallinckrodt Chemical Works, St. Louis, Mo. Dimethyl sulfoxide (DMSO) Spectroquality, was obtained from Matheson, Coleman & Bell, Los Angeles, Calif. Absolute ethanol (Gold Shield) was purchased from Commercial Solvents Corp., Terre Haute, Ind.

Bacterial strains and mutant selection. The *E. coli* K-12 strain E102 was the parental strain for these studies. It is HfrC, *met*$^-$, *tsx*$^+$, *str*$^-$, *lac*$^+$ ($I^+Z^+Y^+$), (λ).

The mutant strains described in this study were obtained from strain E102 after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (100 μg/ml, 30 min at 37 C) according to the procedures of Adelberg, Mandel, and Chen (1). The mutagenized culture was suspended in 1% glycerol-G-salts medium containing 0.3 M ethanol, allowed to grow for 6 h, and then adjusted to 0.82 M ethanol at a density of 10^6 bacteria per ml. These cultures were allowed to grow to a density of 10^8 bacteria per ml and diluted to 10^5 bacteria per ml in 0.82 M ethanol, and growth was continued. The subculturing procedure was repeated three times. Samples of the resulting population were plated on L-broth agar and incubated overnight.

The colonies appearing on the plates fell into two size classes; one class was approximately the same size as the parent and the other class was approximately 1/10 that size. Twenty colonies of each size class were picked, restreaked on L-broth agar plates to verify the colony size, and tested for growth response to ethanol in liquid culture. Bacteria which formed large colonies were designated L (strain L9L100 is a member of this class) and those which formed small colonies were designated S. Strain S9L100 is typical of the small colony formers. The growth properties in response to ethanol of strains S9L100 and L9L100 are described below. Both strains have all the genetic markers of the parent.

Medium and growth conditions. The minimal medium employed in this study was G-salts supplemented with a carbon source (10 mg of glycerol or lactose per ml, as indicated) and 0.1 mg of methionine per ml. The G-salts solution was composed of 3.5 g of K_2HPO_4, 1.5 g of KH_2PO_4, 0.32 g of NH_4CL, 0.10 g of $MgSO_4$, and 1 mg of $FeCl_3 \cdot 6H_2O$ in 1 liter of water. L-broth agar plates (5 g of NaCl, 1 g of glucose, 5 g of yeast extract, 10 g of tryptone, 15 g of agar per 1 liter of H_2O) were used routinely. In all experiments, cultures were grown at 37 C with vigorous agitation. Turbidity was measured at 350 nm (1-cm light path, Beckman DB spectrophotometer).

Permease assay. Since the rate of hydrolysis of ONPG in vivo by intracellular β-galactosidase (EC 3.2.1.23) is limited by the rate of entry of ONPG through the β-galactoside permease (*lac Y* gene product or M protein), this rate provides a measure of the facilitated diffusion by the permease (5, 7, 13). The rate of appearance of orthonitrophenol was followed continuously in a Gilford recording spectrophotometer with thermostatted cuvette chamber. Bacterial suspensions were prepared for assay by washing the bacteria twice by sedimentation and suspending in G-salts, 10^{-3} M Cleland's reagent, 50 μg of chloramphenicol per ml, and 10^{-2} M NaN_3. For the permease assay, 1 ml of bacterial suspension was added to a cuvette containing 1 ml of a solution of ONPG in 0.15 M sodium phosphate buffer, pH 7.0. The rate of hydrolysis of ONPG in vivo in the presence of the permease inhibitors, TDG or formaldehyde, was measured in parallel runs in which the inhibitor was added to the ONPG solution prior to the addition of bacteria. When assays were performed in the presence of organic solvents, the solvents were premixed with the ONPG solution before the addition of the bacterial suspension. Unless otherwise noted, all assays were at 28 C. Results are reported as nanomoles of ONPG hydrolyzed per minute at A_{350} bacteria per ml of assay.

β-Galactosidase assay and determination of the induction level. The β-galactosidase content of the bacterial culture was measured in a lysed sample. A sample of the bacterial culture of known turbidity was suspended in G-salts with 10^{-3} M Cleland reagent and treated with toluene and *N*-lauroyl sarcosinate (7). The β-galactosidase activity was measured by the rate of hydrolysis of a 2 mM ONPG solution as described above.

Fully induced cultures of E102 (grown on 1% glycerol G-salts, 10^{-4} M IPTG for at least five generations) were found to hydrolyze ~900 nmol of ONPG per min at A_{350} bacteria per ml of assay volume. The induction level for a culture is defined as the rate of ONPG hydrolysis in the lysed extract relative to that of the parent strain at equivalent turbidity (bacterial mass). The maximum induction level in the mutant strain S9L100 grown and induced in the absence of ethanol is about 35% of the wild-type level. When S9L100 is grown in 0.35 M ethanol, however, the level of induction is nearly 100%. The reason for the low level of induction of strain S9L100 grown in the absence of ethanol is under investigation.

RESULTS

Physiological effects of ethanol on wild-type E. coli. Three aspects of the effect of ethanol on the growth of *E. coli* have been studied: reduction of growth rate, blockage of cell division, and killing. The results presented below refer to the K-12 strain E102 grown in 1% glycerol-G-salts medium at 37 C. Similar results have been obtained with three other *E. coli* K-12 strains.

Growth rate reduction. The addition of ethanol to an exponentially growing culture of E102 causes an immediate decrease in the growth rate. The result of a typical experiment is shown in Fig. 1. Since growth continues to be exponential for at least three doublings after

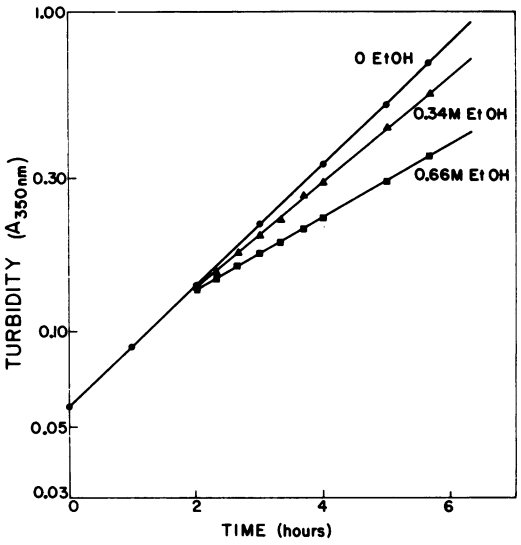

FIG. 1. *Effect of ethanol on the growth of E102. Samples of an exponential culture of E102 growing in 1% glycerol-G-salts were treated with ethanol at the indicated time. This was done by transferring a 25-ml sample of the growing culture to a prewarmed 125-ml screw-top culture flask and quickly mixing absolute ethanol into the culture. Symbols: ●, no ethanol added; ▲, 0.34 M ethanol (0.5 ml of absolute ethanol added); ■, 0.66 M ethanol (1.0 ml of absolute ethanol added).*

addition of ethanol at concentrations up to 0.80 M, growth rate constants have been calculated from data such as those shown in Fig. 1. The growth rate constant, α, decreases almost linearly as a function of ethanol concentration to approximately 50% of the normal growth rate at 0.70 M ethanol, and then rapidly decreases to $\alpha = 0$ at approximately 1.10 M ethanol (Fig. 2).

Blockage of cell division. Although growth after shift to ethanol continues to be exponential at rates dependent on the concentration of ethanol, bacterial morphology is altered when viewed by phase microscopy. The bacteria grow predominantly as filaments and chains at ethanol concentrations above 0.50 M. The formation of filaments and chains is reflected in the differential plot (Fig. 3) of colony formers versus turbidity (mass), in which it can be seen that growth in 0.58 M ethanol results in a decreased colony-forming unit per mass unit.

At 0.82 M ethanol, growth continues for almost exactly one mass doubling and stops. However, there is no increase in the number of colony formers during this mass doubling (Fig. 4). When these cells are examined with phase microscopy, almost all the cells appear either undivided with twice the length of a normal bacterium or as cells in which the cross wall has formed but separation has not occurred. At ethanol concentrations exceeding 0.82 M, the mass increase stops at less than a doubling.

Killing. Over an ethanol concentration range between 1.1 and 1.4 M, neither a mass increase nor a significant loss of viability (colony formers) is observed. At 1.60 M ethanol and above, rapid loss of viability occurs. This killing appears to be exponential with single-hit kinetics (Fig. 5). Killing by 1.60 M ethanol, however, is not observed when bacterial growth is stopped by starvation for a carbon source prior to the addition of ethanol.

Reversibility of the effect of ethanol on growth. The reduction in the growth rate by the addition of ethanol is rapidly reversed when ethanol is removed from the culture. The kinetics of this reversibility are shown in Fig. 6. In these experiments, the ethanol-treated cul-

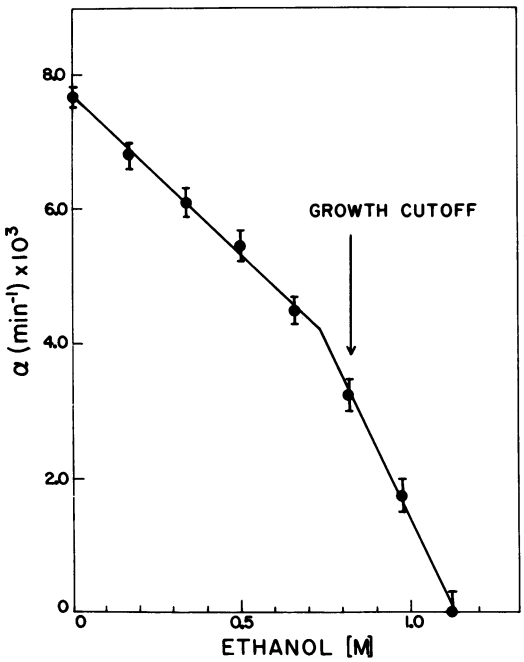

FIG. 2. *Effect of varying ethanol concentration on the growth rate constant, α, of E102. Growth rates were determined from experiments such as shown in Fig. 1. The growth of E102 in the presence of ethanol concentrations up to 0.82 M continues exponentially indefinitely, and, therefore, the calculated rate constant is meaningful. At concentrations of ethanol of 0.82 M and above, growth stops at a mass doubling or less. At these higher ethanol concentrations, a growth-rate constant was calculated by tacitly assuming the initial growth to be exponential. (For example, see Fig. 4.)* $\alpha = \ln(A_{350}[t_2]/A_{350}[t_1])/t_2 - t_1$. *Error bars are ±1 standard deviation.*

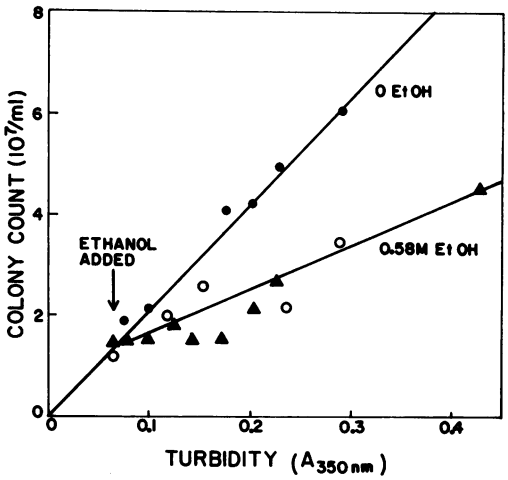

FIG. 3. *Effect of 0.58 M ethanol on the production of colony-forming centers of E102. The experiment was performed as in Fig. 1 except that samples were also plated on L-broth agar to determine the number of colony formers. The data are presented as a differential plot. Each point represents the average of triplicate platings. The symbols ▲ and O correspond to duplicate experiments.*

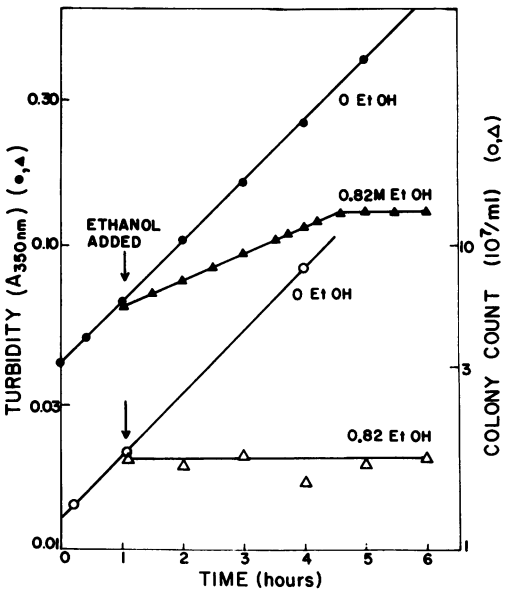

FIG. 4. *Effect of 0.82 M ethanol on the growth of E102. The experiment was performed as in Fig. 1. Symbols: ●, O, no ethanol added; ▲, △, 0.82 M ethanol. Closed symbols are turbidity measurements, open symbols are colony numbers.*

tures were diluted fivefold to rapidly reduce the ethanol concentration with minimum manipulation.

The growth of a culture of E102 treated with

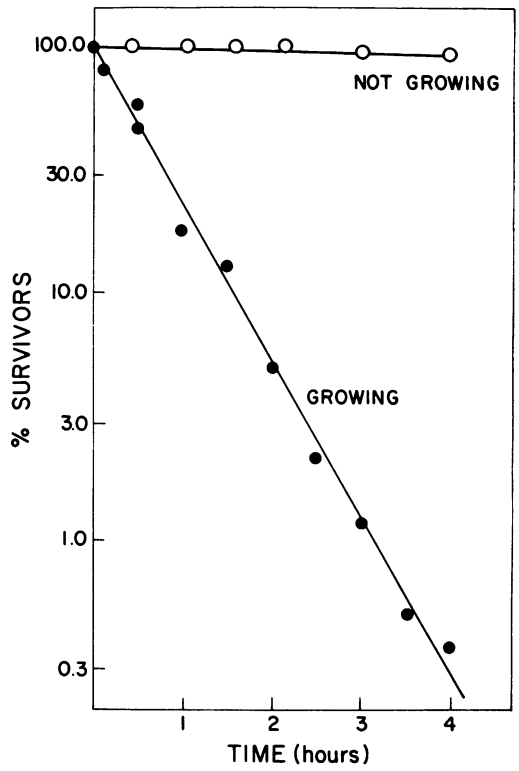

FIG. 5. *Killing of E102 by 1.6 M ethanol. Ethanol was added to (i) an exponentially growing culture of E102 in 1% glycerol-G-salts medium, or to (ii) a culture of E102 suspended in G-salts alone for 60 min before ethanol addition. Samples were plated on L-broth agar to determine colony number. The results are plotted as the fraction of survivors of the initial (0 time) population. Each point is the average of triplicate platings. Temperature was 37 C.*

0.5 M ethanol and diluted to 0.1 M ethanol is shown in Fig. 6A. After a short lag, the culture assumed the growth rate of a culture treated with 0.1 M ethanol. Although this lag period is slightly variable from experiment to experiment, it is not dependent upon the duration of the ethanol treatment.

Figure 6B shows the result of a similar experiment performed with 0.82 M ethanol, a concentration of ethanol which stops growth of E102 after a mass doubling. As in the previous experiment, dilution of the ethanol-treated culture results in a short, variable lag period followed by growth at the higher rate. Particularly striking in this experiment was the lag and resumed growth when the dilution was made after the culture had reached the plateau. After 1-h lag at the plateau (α = 0), the appropriate growth rate was abruptly resumed.

Miscoding. In vitro, ethanol causes errors in

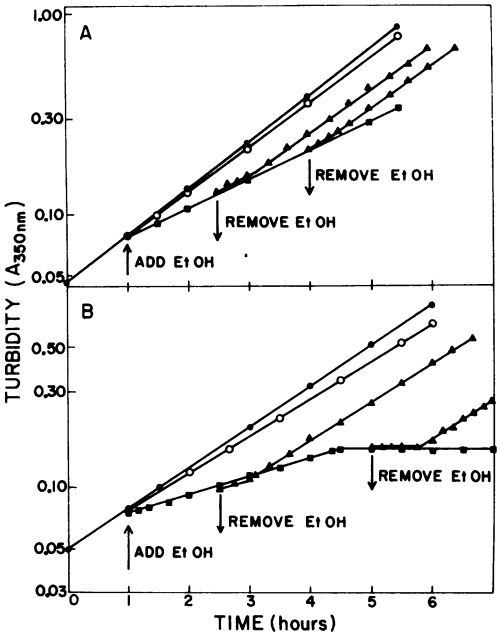

FIG. 6. *Reversibility of the effect of ethanol on the growth of E102. The experiments were performed as in Fig. 1. At the indicated times, the cultures containing ethanol were diluted 1:5 with prewarmed media containing no ethanol. Correction has been made for the dilution factors. A, Reversibility in 0.5 M ethanol. Symbols: ●, no ethanol; ○, 0.1 M ethanol (control for dilution); ■, 0.5 M ethanol; ▲, 1:5 dilution of culture growing in 0.5 M ethanol. B, Reversibility in 0.82 M ethanol. Symbols: ●, no ethanol; ○, 0.16 M ethanol; ■, 0.82 M ethanol; ▲, 1:5 dilution of cultures growing in 0.82 M ethanol.*

protein synthesis (20), and such ethanol-induced errors may also occur in vivo (3). Thus, a trivial explanation for the effects of ethanol on growth is that ethanol induces miscoding, and the accumulation of defective protein interferes with normal growth events. Since streptomycin can suppress phage T4 amber mutants (17), we asked whether ethanol can similarly suppress ambers. Our approach was to infect our wild-type strain E102 with a series of T4 amber mutants (a gift of F. Stahl), expose the infected cultures to 0.5 M ethanol, and assay the cultures for phage at various times after infection. We observed no amber phage production, whereas wild-type phage developed normally under identical conditions in which growth rate of the host (E102) is reduced by about 40%. Thus we conclude that, if ethanol does induce miscoding, it does not do so with a high enough frequency either to suppress amber mutations or to impair normal phage development.

Substrate permeation and nonspecific leakage. It is possible to imagine that ethanol disrupts the systems for the transport of carbon source and required amino acid and thus makes them rate limiting for growth.

To test the above hypothesis, we have taken two experimental approaches. In the first approach, we varied the carbon source (glycerol or lactose) and the required amino acid (methionine) over a 100-fold range. In all cases, the growth rate in ethanol was not significantly affected by changes in the external concentrations of these metabolites.

In the second approach we examined the effect of ethanol on a known transport system, the lactose (*lac*) permease. If the inhibition by ethanol of growth rate on lactose results from an effect of ethanol on the *lac* permease to give a reduced rate of entry of lactose into the bacteria, one would expect the inhibition of growth rate by ethanol to be greater for cells with lower levels of *lac* permease. To prepare bacteria with various levels of *lac* permease, we used the observation that lactose is an inhibitor of induction by IPTG (Suzanne Bourgeois, personal communication). The level of *lac*

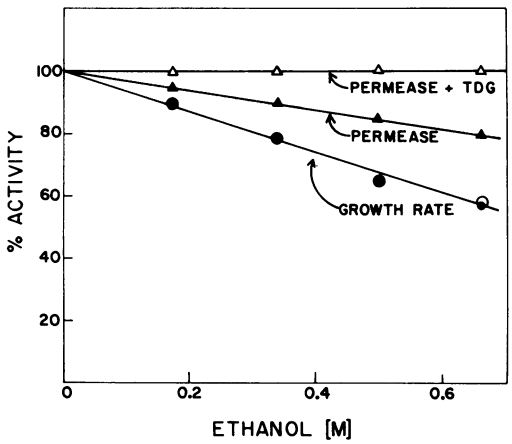

FIG. 7. *Effect of ethanol on the growth rate on lactose and on the activity of lac permease of strain E102. Growth rates in response to ethanol were determined as in Fig. 2. The media, however, were G-salts with 10^{-4} M IPTG and either 0.1% lactose or 1% lactose as the sole carbon source. The growth rates of E102 in 0.1% and 1% lactose were identical; the doubling time in the absence of ethanol was 60 min. Permease activity was measured in a fully induced culture of E102 grown in 1% glycerol-G-salts medium. The assays were performed at an external ONPG concentration of 2 mM at 37 C. Both growth rates and permease activity are reported relative to the values found in the absence of ethanol. Symbols: ●, growth rate in 0.1% lactose medium; ○, growth rate in 1% lactose medium; ▲, permease activity; △, permease activity in the presence of 20 mM TDG.*

permease was varied by growing the bacteria in 10^{-4} M IPTG at various concentrations of lactose.

The effects of ethanol on the growth rate on lactose for cultures having two different levels of *lac* operon induction are presented in Fig. 7. The levels of induction in cultures grown on 0.1% lactose plus IPTG and 1.0% lactose plus IPTG were 50% and 25%, respectively, of the level of induction in a 1% glycerol plus IPTG-grown culture. As is the case for growth in glycerol, there is a linear reduction in growth rate with increase in ethanol, and the inhibition of the growth rates are identical at the two levels of induction.

The effects of ethanol on the *lac* permease are also presented in Fig. 7, where the rate of entry of ONPG (measured by the rate of hydrolysis of ONPG by whole cells grown in the absence of ethanol) is given as a function of the concentration of ethanol present in the assay. The activity of the *lac* permease decreases linearly with increasing ethanol concentration. Further, ethanol does not produce significant "leakiness" since the entry of ONPG by non-specific mechanisms (given by the rate in the presence of TDG, an inhibitor of permease) remains unchanged.

At an ethanol concentration of 0.66 M, the growth rate on lactose at either of the two levels of induction was reduced by 45%, whereas permease activity decreased by only 20%. Since the levels of induction in the two cultures differed by a factor of two and yet both have identical growth rates, it is unlikely that the ethanol inactivation of the transport system is responsible for the reduced growth rate.

Ethanol-resistant mutants. Mutants of E102 were selected (see Materials and Methods) that could grow indefinitely at 0.82 M ethanol, 1% glycerol-G-salts medium. None of these mutants were able to grow indefinitely at concentrations of ethanol exceeding 0.85 M. These mutants fall into two distinct classes. One class, designated L, grows at maximum rate in the absence of ethanol, and the growth rate is unaffected by concentrations of ethanol up to 0.35 M. At ethanol concentrations exceeding 0.35 M, the growth rate drops nearly linearly with increasing ethanol concentration (Fig. 8). The other class, designated S, grows more slowly than the parent strain E102 in the absence of ethanol; but in the presence of 0.35 M ethanol, the growth rate is increased by nearly 30%, bringing it up to the rate of E102 in the absence of ethanol (Fig. 8). We concentrated our study on mutant strain S9L100—a typical example of the second class of mutants. As yet, we have made no further study into the properties of the L-class mutants.

S9L100 grows at all ethanol concentrations from 0 to 0.82 M as a mixture of filaments, chains, and abnormally short cells as well as cells of normal morphology, although larger in diameter than the parent strain. It appears that division occurs at random. The heterogeneity in morphology exhibited by S9L100 in the absence of ethanol is similar to the parent E102 grown in 0.60 M ethanol. This heterogeneity decreases significantly when the mutant bacteria are grown near their growth rate optimum in 0.35 M ethanol and reappears with growth at higher ethanol concentrations.

Killing. No significant loss in viability is observed when S9L100 (actively growing or not growing) is exposed to 1.6 M ethanol, an ethanol concentration which kills the parent strain exponentially.

Characterization of the S9L100 mutation. The transport systems of bacteria have been shown to be sensitive to changes in membrane

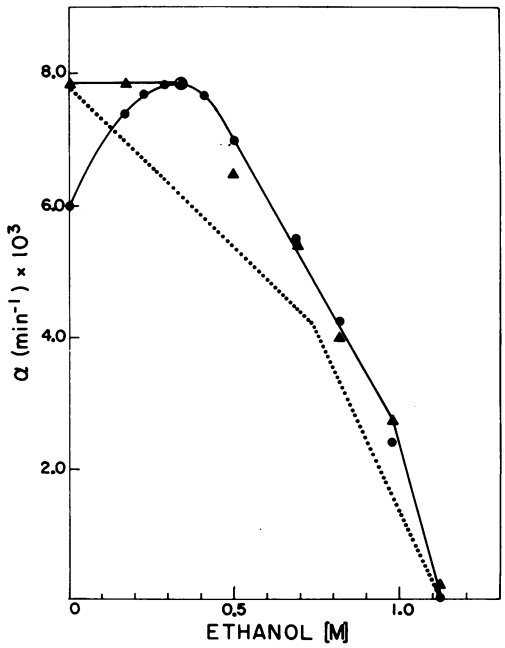

FIG. 8. *Effect of varying ethanol concentrations on the growth rate constant of mutant strains S9L100 and L9L100. Cultures of each strain were grown in 1% glycerol, G-salts, 0.35 M ethanol. When the cultures were in the exponential growth phase, the bacteria were pelleted and resuspended in 1% glycerol-G-salts media containing the indicated ethanol concentrations. Growth rate constants were calculated as described in Fig. 2. Symbols: ▲, strain L9L100; ● strain S9L100. (.....) parent strain E102 from Fig. 2.*

structure (10, 14, 21). We used measurements of the activity of the lactose permease as a probe to screen our mutants for putative changes in membrane organization. A detailed study of the effects of solvent perturbation on the *lac* permease and other transport systems in E102 and S9L100 will be presented elsewhere (manuscripts in preparation). We will present here those aspects of the *lac* permease that lead us to conclude that the mutation in S9L100 concerns a change in the membrane.

The kinetics of ONPG hydrolysis in vivo in E102 are shown in Fig. 9A. In the absence of inhibitors of *lac* permease, the rate of hydrolysis (which is a measure of the transmembrane transport) is the typical hyperbolic function of external ONPG concentration. Such concentration dependence is indicative of facilitated diffusion. The *lac* permease inhibitors TDG and formaldehyde block this facilitated process, and the rate of hydrolysis becomes a nearly linear function of the external ONPG concentration. Linear kinetics are characteristic of passive diffusion across the membrane and are the same as the kinetics of hydrolysis of ONPG in vivo in a Z^+Y^- mutant. (Possible mechanisms for TDG and formaldehyde inhibition are presented in a review article on the *lac* permease by E. P. Kennedy [6].)

The kinetics of ONPG hydrolysis in vivo by S9L100 grown and induced in the absence of ethanol are shown in Fig. 9B. In the absence of inhibitor, the rate dependence on external ONPG concentration is similar to that of E102. However, in the case of S9L100, unlike E102, TDG and formaldehyde do not destroy the facilitated process as they do for E102. The inhibition in the case of S9L100 can be overcome at high external ONPG concentrations unlike with E102. Nonetheless, another property of the *lac* permease, active transport as measured by the accumulation of ^{14}C-thiomethyl-β-D-galactoside (assayed in the absence of azide) is completely inhibited by TDG in S9L100 as well as in E102.

To establish that the entry of ONPG in the presence of TDG is indeed via the *lac* permease in S9L100 grown in the absence of ethanol, experiments were performed with bacteria preinduced to various levels with IPTG (data not shown). In the case of E102, the rate of hydrolysis by whole cells of ONPG in the presence of TDG is essentially independent of the level of induction but proportional to the total number of cells. This demonstrates that the residual entry of ONPG after TDG inhibition is not a permease-mediated process in E102. In contrast to this result, for S9L100

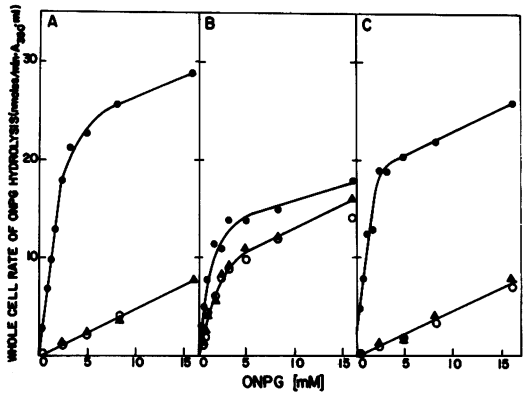

FIG. 9. *Kinetics of in vivo ONPG hydrolysis (28 C) by strains E102 and S9L100. A, Parent strain E102 grown in 1% glycerol-G-salts, 10^{-4} M IPTG. Rate of hydrolysis in lysed sample: 820 nmol of ONPG per min per A_{550} bacteria per ml. B, Strain S9L100 grown in 1% glycerol-G-salts, 10^{-4} M IPTG. Rate of hydrolysis in lysed sample: 300 nmol of ONPG per min per A_{550} bacteria per ml. C, Strain S9L100 grown in 1% glycerol-G-salts, 10^{-4} M IPTG, 0.35 M ethanol. Rate of hydrolysis in lysed sample: 732 nmol of ONPG per min per A_{550} bacteria per ml. Symbols: ●, Assay in the absence of inhibitor; ○, assay with 20 mM TDG; ▲, assay with 10 mM formaldehyde.*

grown in the absence of ethanol, the rate of hydrolysis of ONPG in the presence of TDG is directly proportional to the level of induction, as would be expected if entry is proportional to the level of permease.

When S9L100 is grown and induced with IPTG at concentrations of ethanol near the growth rate maximum, however, the permease now seems to behave like that in wild-type E102. The ability of TDG and formaldehyde to block the facilitated diffusion of ONPG in S9L100, grown at optimal concentrations of ethanol, is entirely restored (Fig. 9C).

To demonstrate that the mutation responsible for the altered sensitivity to TDG by *lac* permease in S9L100 is not in the *lac Y* gene itself, we made P1 phage stocks on S9L100 and transduced the Y^+ allele from S9L100 into a Y^- strain. All Y^+ recombinants tested exhibited normal TDG inhibition of ONPG-facilitated entry.

As further evidence that the defect in S9L100 which gives rise to altered permease function is not the result of a secondary mutation of the *lac* permease, we selected spontaneous revertants of S9L100 that exhibit the parental growth response to ethanol. These revertants occur with a frequency of $\sim 10^{-7}$. They appear identical to the parental strain in their growth-rate dependence on ethanol, morphology, and sensitivity to ethanol killing. The *lac* transport

system in these revertants is identical to that of the parental strain, exhibiting the normal TDG inhibition of facilitated diffusion.

Thus it appears that the unusual behavior of the *lac* permease in S9L100 is not due to a change in the *Y* gene product. We tentatively conclude that the altered specificity arises from the interactions between a "normal" permease and a mutated membrane in S9L100 grown in the absence of ethanol. The altered interactions leading to altered activity may be due either to a change in the lipid phase itself or to the assembly of the M-protein lipid complex.

We investigated the effect of other organic solvents on the growth and transport functions of E102 and S9L100, and the details of these studies will be presented elsewhere. In general, S9L100 is only slightly more resistant to longer carbon chain alcohols than is E102, although the higher alcohols do not significantly stimulate the growth of S9L100. Strain S9L100 is neither stimulated nor resistant to phenethyl alcohol, an agent which appears to affect the integrity of the *E. coli* membrane (18).

Methanol and DMSO, both of which decrease the growth rate of E102, stimulate the growth of S9L100. The maximum growth rate of S9L100 occurs at a methanol concentration of ~0.45 M or at a DMSO concentration slightly greater than 0.50 M.

These observations suggest that mutant strain S9L100 is responding to a general perturbation produced by these solvents and not responding to some unique property of ethanol.

DISCUSSION

We have described the effects of ethanol on some growth properties of the wild-type *E. coli* K-12 strain E102. We have also examined the effects of ethanol on three other *E. coli* K-12 strains with similar results (data not presented). Thus we are confident that the immediate and reversible depression of the growth rate and the disruption of the bacterial division process by ethanol are not unique to E102.

We have also described an ethanol-resistant mutant, S9L100, derived from E102. This mutant strain exhibits pleiotropic growth defects which are relieved by the presence of ethanol, methanol, or DMSO. This mutant also seems to have an altered *lac* permease function which is not due to a mutation in the *Y* gene itself. Evidence will be presented elsewhere that the activities of the *lac* permease and β-glucoside transport systems in the mutant are resistant to these organic solvents (manuscript in preparation). These observations are consistent with the notion that the mutant strain S9L100 has an altered membrane.

At this point we have presented no direct evidence that the biological consequences of ethanol exposure are directly related to a perturbation of the membrane. It is certainly to be expected that ethanol and other such solvents affect cellular functions unrelated to membrane function per se. But the fact that mutants can be generated which simultaneously show altered growth responses to ethanol and altered membrane functions suggests that the membrane may be a common target site of the ethanol perturbation.

Although we have found revertants of S9L100, with a frequency of 10^{-7}, which completely restore the wild-type response to ethanol and which simultaneously lose all mutant characteristics, we have not yet rigorously established that the pleiotropic properties of the mutant are due to a single mutational event. Further characterizations of the ethanol-resistant mutants, both genetic and biochemical, must be made before we can draw any firm conclusions. Nevertheless, we can consider some general hypotheses that may explain the effects of ethanol on bacterial growth.

One possible hypothesis to explain the reduction of growth rate of normal bacteria in response to ethanol is based on the report that ethanol can produce miscoding in protein synthesis in vitro (20) and possibly in vivo (3). The strongest evidence against miscoding as a primary cause of the ethanol effects comes from the fact that, upon addition of ethanol, there is an almost instantaneous reduction in the steady-state growth rate. The miscoding hypothesis would predict a gradual reduction. Further evidence against the miscoding hypothesis comes from the observation reported here that there is no suppression of T4 amber mutants by ethanol.

One hypothesis we consider is that ethanol affects the growth of bacteria as a structural perturbant of the membrane but that the major consequence of the postulated perturbation is a disruption of the transport systems and of the integrity of the permeation barrier. Thus growth could be affected by decreased availability of metabolites due to inactivation of the transport systems or to leakage of the internal pools through a porous membrane.

We have presented evidence that the ethanol inactivation of the *lac* transport system is not responsible for the growth-rate reductions (Fig.

7). Furthermore, the effect of ethanol on the growth rate of wild-type bacteria is similar for cultures grown on either lactose or glycerol as the sole carbon source. Thus it seems unlikely that the growth rate is limited by the transport of carbon source in general. We have also shown (Fig. 7) that ethanol treatment does not increase the passive rate of ONPG influx—i.e., ethanol does not make the membrane "leaky" to molecules the size of ONPG. The membrane may, however, have become permeable to smaller molecules or inorganic cations. We are exploring this possibility.

The mechanism and degree to which agents such as ethanol perturb membrane organization is subject to direct experimental investigation. At present, however, we can only speculate on the nature of the ethanol perturbation. The current understanding of membrane organization (see Singer for a review [19]) has led us to consider two simplistic mechanisms by which perturbants can alter the organization of the phospholipid phase. On the one hand, the perturbant may intercalate into the hydrophobic regions of the membrane and directly disrupt the interactions between the hydrocarbon chains of the phospholipids, or protein components, or both. On the other hand, a perturbant may alter the properties of the aqueous phase and thereby alter the organization of the membrane at the aqueous-phospholipid interface.

One effect of the addition of alcohols to the aqueous phase would be a reduction of the dielectric strength (2). This should lead to altered charge interactions at the phospholipid surface and, in particular, increase the binding of divalent cations to the polyphosphate surface of the membrane. An increase in Ca^{2+} binding to red-blood-cell membranes in response to the addition of alkanols has been observed by Seeman et al. (16). Although the concentrations of ethanol we use would provide only a small reduction in the dielectric strength of the milieu, the solubility of ethanol in the lipid phase may allow a relatively high concentration of ethanol to exist at the aqueous-phospholipid interface. We are exploring the possibility that ethanol, and other solvents to which the mutant S9L100 is resistant, affect membrane organization primarily by altering the charge interactions at the aqueous-membrane interface.

Since it appears that the bulk of the enzymes for the synthesis of phospholipids (12), cell wall (4), and outer membrane components (9) are associated with cytoplasmic membrane, a perturbation of the membrane structure by general agents such as ethanol could lead to a defect in morphology and the division process itself. Quite possibly, the use of nonspecific membrane perturbants and the characterizations of resistant mutants may provide insight into the role of the membrane in ordering biological processes.

ACKNOWLEDGMENTS

We acknowledge the expert technical assistance of M. Pratt.

This investigation was supported by Public Health Service grant 2 PO1 GM15423 and Training Grant 2 TO1 GM00715, both from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Adelberg, E. A., M. Mandel, and G. C. C. Chen. 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **18**:788–795.
2. Franks, F., and D. J. G. Ives. 1966. The structural properties of alcohol-water mixtures. Quarterly Reviews (London) **20**:1–44.
3. Gorini, L., R. Rosset, and R. A. Zimmermann. 1967. Phenotypic masking and streptomycin dependence. Science **157**:1314–1317.
4. Hardmann, R., J-V. Höltje, and U. Schwarz. 1972. Targets of penicillin action in *Escherichia coli*. Nature (London) **235**:426–429.
5. Herzenberg, L. A. 1959. Studies of the induction of β-galactosidase in a cryptic strain of *Escherichia coli*. Biochim. Biophys. Acta **31**:525–538.
6. Kennedy, E. P. 1970. The lactose permease system of *Escherichia coli*, p. 49–92. *In* J. R. Beckwith and D. Zipser (ed.), The lactose operon. Cold Spring Harbor Laboratory, New York.
7. Koch, A. L. 1964. The role of permease in transport. Biochim. Biophys. Acta **79**:177–200.
8. Mullins, L. J. 1954. Some physical mechanisms in narcosis. Chem. Rev. **54**:289–323.
9. Osborn, M.-J., J. E. Gander, and E. Parisi. 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. J. Biol. Chem. **247**:3973–3986.
10. Overath, P., H. U. Schairer, and W. S. Staffel. 1970. Correlation in *in vivo* and *in vitro* phase transitions of membrane lipids in *Escherichia coli*. Proc. Nat. Acad. Science. U.S.A. **67**:606–612.
11. Pauling, L. 1961. A molecular theory of general anesthesia. Science **134**:15–21.
12. Raetz, C. R. H., and E. P. Kennedy. 1972. The association of phosphatidylserine synthetase with ribosomes in extracts of *Escherichia coli*. J. Biol. Chem. **247**:2008–2014.
13. Rickenberg, H. W., G. N. Cohen, G. Battin, and J. Monod. 1956. La galactoside-permease d'*Escherichia coli*. Ann. Inst. Past. **91**:829–857.
14. Schairer, H. U., and P. Overath. 1969. Lipids containing trans-unsaturated fatty acids change the temperature characteristic of thiomethylgalactoside accumulation in *Escherichia coli*. J. Mol. Biol. **44**:209–212.
15. Seeman, P. M. 1966. Membrane stabilization by drugs: tranquilizers, steroids, and anesthetics. Int. Rev. Neurobiol. **9**:145–221.
16. Seeman, P., M. Chan, M. Goldberg, T. Sanks, and L. Sax. 1971. The binding of Ca^{++} to the cell membrane increased by volatile anesthetics (alcohols,

acetone, ether) which induce sensitization of nerve or muscle. Biochim. Biophys. Acta **225**:185–193.
17. Schwartz, R. D., and V. Bryson. 1970. Streptomycin-induced phenotypic suppression of hydroxylamine-induced nonsense mutants in the rII A cistron of bacteriophage T4. J. Virol. **5**:22–26.
18. Silver, S., and L. Wendt. 1967. Mechanism of action of phenethyl alcohol: breakdown of the cellular permeability barrier. J. Bacteriol. **93**:560–566.
19. Singer, S. J. 1971. The molecular organization of biological membranes, p. 145–222. *In* L. I. Rothfield (ed.), Structure and function of biological membranes. Academic Press Inc., New York.
20. So, A., and E. W. Davies. 1964. The effect of organic solvents on protein biosynthesis and their influence on the amino acid code. Biochemistry **3**:1165–1169.
21. Wilson, G., S. P. Rose, and C. F. Fox. 1970. The effect of membrane lipid saturation on glycoside transport. Biochem. Biophys. Res. Commun. **38**:617–623.